

IN THE TITLE

Please replace the Title (page 1, line 1) as follows:

IN SITU IDENTIFICATION OF TARGET STRUCTURES E.G. *IN VIVO*

C₂ SELECTION METHOD FOR A PHAGE LIBRARY

IN THE SPECIFICATION:

Please amend the specification as follows:

Kindly replace the paragraph beginning at page 2, line 18, with the following:

C₃ The purpose of the present invention is to provide an extension of the application of phage technology for the selection of antibodies to complex antigens, thus making it generally applicable to identify antibodies directed against a number of important and/or novel target antigens and epitopes which are not accessible in *in vitro* culture systems, which would facilitate identification and dissection of antigens which are exclusively expressed *in vivo*. Of course, the method could generate antibodies to any target structure displayed within the tissue section.

Kindly replace the paragraph beginning at page 3, line 24, with the following:

C₄ FIG 1 is a histogram demonstrating yield (output/input of colony forming units, CFU) of C215 (black bars) and D1.3 (white bars) scFv phage and enrichment of specific phage at various amount of phage added in a volume of 100 μ l. The ratio, 1:15, of the C215 and D1.3 phage in the dilution series was kept constant.

Kindly replace the paragraph beginning at page 5, line 5, with the following:

~ In order to make the method more effective and specific the steps (a) through (c) as well as the steps (a) through (d) can be repeated. The amplification of bound binding structures can be obtained by means of synthesis in growing bacterial cells, PCR (polymerase chain reaction) synthesis, and chemical synthesis. Thus, monoclonal antibody, single-entity, homogenous, uniform and/or other binding structures can be isolated and/or amplified from the second, or third, or fourth etc enriched library.

Kindly replace the paragraph beginning at page 6, line 8, with the following:

~ The displayed target structure can be obtained within a set of desired as well as undesired displayed target structures. In either case, bound structures or unbound structures are recovered in dependence of the selection intended. A selection system can thus be a combination of a tissue phenotype subtractive approach, i.e. the use of both positive and negative selection, with the use of different types of libraries, e.g. large naive or semi-synthetic libraries.

[Kindly replace the paragraph beginning at page 6, line 15, with the following:]

~ The target structure displayed *in vivo* and/or *in situ* and representing authentic *in vivo* and/or *in situ* phenotype is preferably obtained from tissue sections by a histological technique which comprises freezing and/or fixing, and sectioning a

tissue sample. Such sections from a frozen tissue sample closely represent the original phenotype of all components at the moment the sample was frozen.~

[Kindly replace the paragraph beginning at page 6, line 21 with the following:]

~In practice, a tissue is frozen *in vivo* immediately after surgical removal from a human being or an animal, and the displayed target structure is localized *in situ*. For the skilled man, there is no principal difference between a frozen tissue section and the same tissue *in vivo*.~

Kindly replace the paragraph beginning at page 7, line 1, with the following:

~from cells. The secretions can be secreted actively as well as passively. Actively secreted secretions are, for example, cytokines.~

[Kindly replace paragraph beginning at page 7, line 3, with the following:]

C7 ~The first library of binding structure according to the invention can be a naive, synthetic, or semi-synthetic antibody library. It can also be a combinatorial and/or preselected library, the combinatorial and/or preselected library preferably being a library produced by immunization against one or more displayed target structures. However, the combinatorial and/or preselected library can also be a chemical library.~

[Kindly replace the paragraph beginning at page 7, line 9, with the following:]

According to the invention, a first library or one or several binding structures is linked to genetic and/or other identifying information. Preferably, the linkage between such binding structures and genetic and/or other identifying information comprises particles of a filamentous phage or of any other virus. The linkage can also comprise polysomes or coded beads, i.e. beads identified by means of coding.

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Kindly replace the paragraph beginning at page 7, line 22, with the following:

C₈ Pasqualini and Ruoslahti (*Nature* 380: 364-366, 1996) disclose a technique for selection of phage particles. Anyhow, this technique is clearly distinguished from the present invention in that it is profoundly restricted to selection towards the intravascularly accessible endothelial cell surface.

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Kindly replace the paragraph beginning at page 8, line 1, with the following:

C₉ human peripheral blood samples were obtained from Lund University Hospital and Malmö General Hospital, Sweden. Human melanoma cell lines FM3 (kind gift from Dr. Jesper Zeuthen) and FMEX (ATCC) were used in FACS analyses. All tissues were snap-frozen in isopentane, cooled in liquid nitrogen and stored at 70°C until cryostat sectioned. Six µm frozen section 3x4 mm wide were mounted on slides and air dried overnight (o/n).

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Kindly replace the paragraph beginning at page 8, line 8, with the following:

The different phagemid vectors, all based on pBR322, were equipped with ampicillin or chloramphenicol resistance genes and a gene for the scFv-M13 pIII (residues 249-406) fusion protein expressed from the *lac* or the *phoA* promoter with the secretion directed by either the *ompA* or the ST II signal peptide. An amber stop codon allowed for production of soluble scFv molecules in non-suppressor strains. The fusion proteins contained either a recognition site for His64Ala subtilisin (Ala- Ala-His-Tyr) (17) or Restriction Protease Factor Xa (Ile-Glu-Gly-Arg) situated amino terminal of pIII. An expression plasmid vector carrying a kanamycin resistance gene and the *lac* promoter was constructed for cassette insertion of scFv fragments in frame fusion with the superantigen staphylococcal enterotoxin A (SEA). The model scFv antibody constructs were derived from the C215 antibody directed to a well characterized epithelial antigen (the affinity for Fab binding is 2.3 nM) (24), and from the D1.3 anti-lysozyme antibody (25). For construction of a scFv antibody library, first strand cDNA was synthesized from total mRNA from lymph nodes of a Cynomolgus Macaque immunized with a suspension of pooled human malignant melanoma metastases mixed with alum adjuvant. Family specific sense primers annealing to the first framework region of human VH (IgG) and VL (lambda, only) genes and antisense primers annealing in the CH1 and CL regions respectively were used for the first PCR. ScFv genes, VL-(Gly4Ser)₃-VH, were assembled and inserted into *MluI* and *XhoI* sites of the phagemid vector. The phagemid pool was transformed to *E. coli* TG-1 cells. Three times 10⁷ primary transformants were

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spread on minimal-agar plates, grown and pooled before superinfected with
M13K07~~+~~

Kindly replace the paragraph beginning at page 9, line 4, with the following:

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Tissue sections were air-dried on slides, fixed in acetone at -20°C for 10 min and rehydrated in 20% fetal calf serum (FCS) in TBS in a humid atmosphere for 1 hour at room temperature. Model or library antibody phage in 100 ml 20% FCS were incubated at 4°C o/n. The slides were washed 6x10 minutes by gentle agitation in 40 ml TBS in 50 ml Falcon tubes. Depending on the elution method, either of the following steps were performed: (i) Washes for 2x5 min in 50 mM Tris pH 7.6, 1 M NaCl and 2 times in 1xPBS pH 7.6. Phage were diluted with 300 µl 0.1 M triethylamine for 15 min and neutralized with 150 µl 1M Tris pH 7.4. (ii) Washes for 2x5 min in 1 M NaCl, 10 mM Tris-HCl, 6 mM CaCl₂, 1 mM EDTA, pH 8.0 (Ala-64 subtilisin buffer) or 100 mM NaCl, 50 mM Tris-HCL, 1mM CaCl₂, pH 8.0 (restriction factor Xa buffer). Bound phage particles were eluted by volume 300 µl 30 mg/ml mutant Ala64-subtilisin for 30 min or volume 300 µl 100 mg/ml restriction factor Xa (New England Biolabs, Beverly, MA) for 2 h. All washing and elution steps were performed at room temperature. Phage titres of incubation solutions and eluates were determined by counting antibiotic resistant colony forming units (CFU)~

Kindly replace the paragraph beginning at page 10, line 5, with the following:

C12 Tissue sections first treated as described above were blocked with avidin 15 min and then with biotin 15 min diluted 1/6. Primary antibodies, scFv or scFv-Sea, were incubated for 1 h, followed by secondary 1 mg/ml affinity purified rabbit antibodies to the ATPAKSE tag peptide or 5 mg/ml rabbit antibodies to SEA for 30 min, and biotinylated goat anti-rabbit Mab diluted 1:1000 (Sigma) for 30 min, StreptABComplex HRP (DAKO) diluted 1:110 in 50 mM Tris for 30 min. Between all steps the sections were washed 3 times in TBS. Antibodies, avidin and biotin were diluted in 20% FCS in TBS. The staining reaction was developed for 8 min in 0.5 mg/ml DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma) dissolved in Tris pH 7.6 with 0.01 percent H₂O₂. The slides were rinsed 10 min in tap water and gradually dehydrated in 70-95 % ethanol and Xylene before mounting in DPX medium (Sigma).

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Kindly replace the paragraph beginning at page 10, line 19, with the following:

C13 FM3 or FMEX melanoma cells 200,000 CELLS in 100 ml 1% BSA per tube, were incubated on ice 1 h with the primary antibody scFv K373-SEA (no primary antibody for the negative control), 30 min with 1 mg/ml rabbit anti-SEA Ig and 30 min with fluorescein-conjugated donkey anti-rabbit Ig (Amersham Life Science) diluted 1:100. Two washes in 1% BSA followed each step. The samples were analyzed by FACS (Becton Dickinson).

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Kindly replace the paragraph beginning at page 13, line 4, with the following:

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Most antigens in a tissue section could be expected to distribute over only a fraction of the total tissue surface area (subpopulations of cells/substructures of tissue). As varying antigen distribution in individual tissue section was difficult to reconstruct for model experiments, a mosaic of antigen negative and antigen positive sections was constructed. Slides covered with 30 tissue sections of Colo205 SCID tumor and human spleen in various proportions were produced. A total amount of 3.6×10^8 scFv C215 phage and 3.0×10^{10} control phage was added per slide. The yield of specific phage increased linearly with an increasing number of antigen positive tissue sections, whilst the yield of scFv D1.3 phage was not effected (fluctuated two-fold or less) by the different proportions of tissues within the experiment (FIG 2). When only antigen-negative (spleen) tissue was applied, the yield of phage of the two populations was similar. Thus, no difference in intrinsic non-specific binding between the phage stocks was seen. Unspecific binding of D1.3 phage did not differ significantly (less than two-fold) between other non-antigen expressing tissue, chosen to represent lipid-rich, epithelial parenchymal and mesenchymal organs (human brain, C. Macaque liver, human spleen and human heart, respectively (data not shown). The effect on enrichment of specific phage by reducing the antigen-expressing surface fraction was also exemplified by a four-fold decrease in yield of specific C215 phage after binding to a primary colorectal cancer biopsy containing epithelial tumor cell areas together with smooth musculature layers and connective tissue of the normal colon components (not shown).

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Kindly replace the paragraph beginning at page 13, line 29, with the following:

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The first selection round, which is aimed at rescue and enrichment of rare specific phage in a large library, is critically dependent on both the specificity of the procedure, i.e. the enrichment factor and the capacity scale of the

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Kindly replace the paragraph beginning at page 15, line 1, with the following:

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and K382, were unique and that all but one clone in the third round had a pattern identical to K378. The predominant representation of the K378 clone in the third round paralleled the increased phage yield at this stage. In a model experiment, the scFv K378 phage was enriched 2490, 575 and 2 times over D1.3 sections of two different melanoma samples and of human spleen (not shown). The scFv antibody genes of K373 and K378 were re-cloned and expressed as fusion proteins with staphylococcal enterotoxin A (SEA) which was used as detection "tag" in immunoassays.

Kindly replace the paragraph beginning at page 15, line 12, with the following:

C₁₇
The scFv K373-SEA fusion protein was demonstrated by FACS analyses to bind to cultured cells of the FM3 (FIG 4A) and FMEX (not shown) melanomas but not to human peripheral blood lymphocytes (FIG 4B). The intensity of the staining indicated that the epitope was strongly and homogeneously expressed on viable cells and thus demonstrated the cloning of a cell surface reactive antibody phage through the use of a tissue-based selection method. The scFv K373-SEA but not

the scFv D1.3-SEA fusion protein strongly and homogeneously stained melanoma cells in section of metastatic melanoma tissue (not shown). By FACS analyses, the K378-SEA fusion protein was found to bind only weakly to FM3 and not to FMEX or PBL. However, in tissue sections of metastatic melanoma this antibody bound strongly to both the melanoma cells and stroma components (not shown).

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Kindly replace the paragraph beginning at page 15, line 26, with the following:

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--A mixture of filamentous phage particles displaying the C215 scFv (3.6×10^9 particles) specific for an antigen expressed in all human epithelia, including small intestine and large intestine (colon) and the 1F scFv displaying phage (1.1×10^8), reactive with only colon epithelium, was applied to frozen sections of small intestine or control (uterus) tissue in order to perform a negative selection step. After overnight incubation at +4°C, unbound

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Kindly replace the paragraph beginning at page 16, line 1, with the following:

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--phages in the supernatant were transferred to sections of colon epithelium in order to perform a positive selection step. After overnight incubation at +4°C, sections were washed six times for 10 min with 50 mM TRIS, pH 7.6 with 0.15 M NaCl. By incubation with Ala64-subtilisin (33 µg/ml) at RT, bound particles were eluted and used to infect *E. coli* DH5alphaF' bacterial cells. Infected bacterial cells were grown as single colonies on agar plates. Colonies were counted and used to estimate the number of phage particles in the eluates. By using different antibiotic resistance

genes for the two scFv phagemid constructs, the number of phage type could be estimated.

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Kindly replace the paragraph beginning at page 16, line 22, with the following:

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According to the method of the invention antibody phage can be directly selected in frozen tissue sections. The concentration of phage applied to the tissue sections influenced non-specific binding and efficiency of positive enrichment. Concentrations higher than $5 \times 10^9/100 \mu\text{l}$ reduced yield of non-specific phage, indicating that a limited number of high-affinity non-specific binding sites could be saturated at these concentrations. This was in contrast to low-affinity non-saturable sites responsible for a relatively constant yield (about 10^{-6} of input) of unspecifically bound phage particles within the high concentration range. Saturation of specific C215 epitopes could not be achieved even at the highest practicable phage con-

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Kindly replace the paragraph beginning at page 17, line 1, with the following:

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--centration, even though only fraction ($<5 \times 10^6$) of the estimated number of available tissue epitopes ($>10^{10}$) were utilized for phage binding. Phages displaying antibody-fragments (about 25 percent of the scFv C215 population by Western blot analysis, not shown) were present in large excess (>2000 -fold) as compared to bound and eluted phage. At the highest phage concentration used, corresponding to $\approx 0.2 \text{ nM}$ of C215 displaying phage, >6 percent of soluble antibody (affinity 2.3 nM) would be bound at equilibrium. This discrepancy suggests that other factors,

e.g. steric hindrance and diffusion limitations, will be important to consider for further improvement of binding efficiency and selection capacity.~

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Kindly replace the paragraph beginning at page 17, line 16, with the following:

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~In single-pass experiments, enrichment of a few hundred times was routinely obtained, as compared to the relatively low enrichment factors (19 at the low end, Table 1) seen before optimization. After optimization, a useful enrichment of antibodies could be achieved even when using heterogenous samples with only 3 percent of antigen-specific surface area, mimicking cell subpopulations/substructures of a tissue (FIG 2). The million-fold enrichment achieved in three selection rounds on tissue sections was comparable to phage selection using cell suspensions (8, 12) and to use of cultured epithelial cells for enrichment of the same scFv C215 phage (not shown) and to pure antigen systems (1). For a given enrichment factor, the probability of rescuing rare specific phage will directly correlate to the number of eluted phage (which is limited by the total capacity of the selection system); this suggests that the high-end of the concentration range investigated (10^{10} - 10^{11} CFU per 100 μ l), should optimally be used.~

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Kindly replace the paragraph beginning at page 18, line 16, with the following:

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~Mabs to melanoma-associated antigens resulting from immunization of non-human primates have previously not been established, although primates have been used previously for tumor-immunization to produce polyclonal antibody reagents